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石　明、嶋千絵子、足立　靖、南野桂三、高橋寛二、池原　進.	マグネット･ビーズを用いた骨髄内骨髄移植法.	第 101 回日本病理学会総会	東京	平成 24 年 4 月 26 日～平成 24 年 4 月 28 日
足立　靖、松添弘樹、川田交俊、高津宏樹、生田寿彦、石田明彦、中野麗香、山内荘作、下　智比古、池原　進.	巨大右心房内血栓症から両肺の肺動脈血栓塞栓症をきたした 1 例	第 58 回日本病理学会秋期特別総会	名古屋	平成 24 年 11 月 22 日～平成 24 年 11 月 23 日
LI Ming, LI Ming, SHI Ming, Ikehara Susumu.	Bone marrow transplantation improved SMP30 expression in the liver of type II diabetes mice.	第 41 回日本免疫学会学術集会	神戸	平成 24 年 12 月 5 日～平成 24 年 12 月 7 日
石井さなえ、島田厚良、稲葉宗夫、李銘、石明、河村則子、武井史郎、千葉陽一、榎戸靖、河内全、細川昌則、池原進	骨髄由来免疫系細胞が健常脳に進入する新たな経路:骨髄内骨髄移植による組織学的同定。	第 53 回日本神経病理学会	新潟	2012.6.30.
島田厚良、石井さなえ、稲葉宗夫、李銘、石明、河村則子、武井史郎、千葉陽一、榎戸靖、河内全、細川昌則、池原進	老化促進モデルマウスにみられる骨髄由来細胞の脳実質へのリクルートの亢進。	第 53 回日本神経病理学会	新潟	2012.6.30.
足立　靖、下智比古、山内壮作、沖垣光彦、梅澤一夫、石明、金子一成、池原進.	微小糸球体病変モデルマウスに対する NF-κB 阻害薬 DHMEQ の効果.	第 102 回日本病理学会総会	札幌	平成 25 年 6 月 6 日～平成 25 年 6 月 8 日
李　銘、石　明、池原進.	SAMP10 マウスの胸腺上皮細胞における Sirt1 発現についての検討 ―骨髄内骨髄移植を用いて―	第 13 回日本抗加齢医学会総会	横浜	平成 25 年 6 月 28 日～平成 25 年 6 月 30 日
Q. Li, H. Hisha, T. Takaki, Y. Adachi, M. Li, J. Kato, M. Inaba, N. Hosaka, M. Maki, S. Ikehara	Transformation potential of bone marrow stromal cells into undifferentiated high grade pleomorphic sarcoma	14th International Congress of Immunology	Kobe, Japan	August 23, 2010～August 27, 2010
Y. Cui, S. Nakamura, M. Shi, Q. Li, M. Li, S. Ikehara	Prevention of premature ovarian failure and osteoporosis induced by irradiation using allogeneic ovarian/ bone marrow transplantation	14th International Congress of Immunology	Kobe, Japan	August 23-27, 2010

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M. Shi, Y. Adachi, Y. Cui, M. Li, Q. Li, S. Yanai, S. Ikehara	Intra-bone marrow injection of donor bone marrow cells suspended in collagen gel retains injected cells in bone marrow, resulting in rapid hemopoietic recovery in mice	14th International Congress of Immunology		
M. Li, M. Inaba, S. Hoshino, K. Okazaki, N. Abraham, S. Ikehara	Amelioration of cognitive ability in senescence-accelerated mouse prone 8 (SAMP 8) by intra-bone marrow-bone marrow transplantation	14th International Congress of Immunology	Kobe, Japan	August 23-27, 2010
S. Ikehara.	A novel BMT technique for the treatment of various currently intractable diseases: From bench to bedsaide	6th International Symposium: Haploidentical Stem Cell Transplantation. 招聘講演	Jerusalem, Israel	September 12-14, 2010
S. Ikehara.→代理で M. Li が発表	Factors involved in aging: mesenchymal stem cells and thymus	Multidisciplinary Conference: Lifestyle and Ageing 招聘講演	Pisa, Italy	October 4-5, 2010
M. Li, M. Shi, N.G. Abraham, S. Ikehara	Amelioration of cognitive ability in senescence-accelerated mouse prone 8 (SAMP 8) by intra-bone marrow-bone marrow transplantation	Multidisciplinary Conference: Lifestyle and Ageing	Pisa, Italy	October 4-5, 2010
S. Ikehara	A revolutionary therapy for the treatment of disorders of hemopoietic stem cells (HSCs) and/or mesenchymal stem cells (MSCs)	The Fourth International Conference on Cell Therapy 招聘講演	Seoul, Korea	November 11, 2010
Susumu Ikehara.	Autoimmune diseases as stem cell disorders: Rationale for normal stem cell transplantation for their treatment.	The 5th Autoimmunity Congress Asia (ACA 2011). 招聘講演	November 17-19, 2011.	Singapore
Ming Li, Ming Shi, Susumu Ikehara.	Improved SMP30 expression in the liver of diabetic mice by stem cell Transplantation. KEYSTONE SYMPOSIA on Molecular and Cellular Biology	Aging and Diseases of Aging	Tokyo, Japan	October 22-27, 2012
Ming Li, Susumu Ikehara.	Prospects for bone marrow transplantation in tolerance induction of organ transplantation.	7th Five-Continent International Symposium on Cardiovascular Disease 招聘講演	Beijing, Cjina	April 19-April 21, 2013.
Sanae Hasegawa-Ishii, Atsuyoshi Shimada, Muneo Inaba, Ming Li, Ming Shi, Noriko Kawamura, Shiro Takei and Susumu Ikehara	Intra bone marrow procedure facilitates entry of transplanted bone marrow cells through the tenia of choroid plexus into brain parenchyma.	19th Annual Meeting of The PsychoNeuroImmunology	San Diego	2012.6.7

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Atsuyoshi Shimada, Sanae Hasegawa-Ishii, Muneo Inaba, Ming Li, Ming Shi, Noriko Kawamura, Shiro Takei and Susumu Ikehara	Enhanced recruitment of bone marrow-derived cells into the brain parenchyma in senescence-accelerated mice.	19th Annual Meeting of The PsychoNeuroImmunology	San Diego	2012.6.7
anae Hasegawa-Ishii, Atsuyoshi Shimada, Muneo Inaba, Ming Li, Ming Shi[5], Shiro Takei, Susumu Ikehara	Selective localization of bone marrow-derived ramified cells in the brain adjacent to the attachments of choroid plexus.	20th Annual PNIRS Scientific Meeting	Stockholm, Sweden	June 5-8, 2013
Akatsuka Y, Yamamura Y, Bleakley M, Hikita J, Hamajima T, Nannya Y, Matsubara A, Riddell SR, Takahashi T, Kuzushima K, Ogawa S.	Identification of novel minor histocompatibility antigens using HAPMAP EBV-LCL panels transduced with restricting HLA cDNA retrovirally.	第 16 回日本遺伝子治療学会総会(ポスター #147) The 16th Annual Meeting-JSGT2010, 2010.	宇都宮	2010 年7月 1 日
小川誠司, 松原亜以子, 鬼塚真, 柏瀬貢一, 真田昌, 南谷泰仁, 赤塚美樹, 佐竹正博, 千葉滋, 佐治博夫, 丸谷悦子, 猪子英俊, 森島泰雄, 小寺良尚, 笹月健彦.	MHC と疾患 GWAS の手法による同種造血幹細胞移植の遺伝学的背景の探索.	第 13 回日本組織適合性学会大会(口演) MHC: Major Histo-compatibility Complex 17 巻 2 号, 141, 2010.	東京	2010 年9月 18 日
Yamamura T, Bleakley M, Hikita J, Matsubara A, Hamajima T, Nannya Y, Takahashi T, Emi N, Morishima Y, Kodera, Y Kuzushima K, Riddell SR, Ogawa S, Akatsuka Y.	Development of an Online Tool to Scan Single Nucleotide Polymorphisms for Identification of Novel Minor Histo-compatibility Antigens.	第 17 回 BMT Tandem Meetings(ポスター #508) Biology of Blood and Marrow Transplantation. 17(2) Suppl.1, pp S335, 2011.	ハワイ	2011 年2月 19 日

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赤塚美樹	マイナー組織適合抗原の重要性.	第 33 回日本造血細胞移植学会(シンポジウム2) 日本造血細胞移植学会総会プログラム・抄録集 pp164 , 2011.	松山	2011 年3月9日
赤塚美樹、山村武史、Bleakley Marie、疋田潤哉、濱島剛、南谷泰仁、松原亜以子、Riddell Stanley、恵美宣彦、小寺良尚、森島泰雄、小川誠司.	HapMap 資源を利用したマイナー組織適合抗原に関わる SNP 同定のためのオンラインソフトの開発.	第 33 回日本造血細胞移植学会(ポスター PS2-128) 日本造血細胞移植学会総会プログラム・抄録集 pp336, 2010.	松山	2011 年3月 10 日
赤塚美樹、森島泰雄、田地浩史、山本一仁、宮村耕一、高橋利忠、小寺良尚、恵美宣彦、葛島清隆.	同種移植後再発予防・治療を目的としたマイナー抗原ワクチン臨床試験(中間報告)(口演 #39).	第 15 回日本がん免疫学会総会 日本がん免疫学会総会プログラム・抄録集 pp17, 2011.	大阪	2011 年 6 月 30 日
赤塚美樹、森島泰雄、田地浩史、山本一仁、宮村耕一、高橋利忠、小寺良尚、恵美宣彦、葛島清隆.	同種移植後再発予防・治療を目的としたマイナー抗原ワクチン臨床試験の中間報告.	第 3 回造血器腫瘍免疫療法研究会学術集会(口演)	別府	2011 年 8 月 21 日
岡村文子、鳥飼宏基、赤塚美樹、三好浩之、吉森 保、葛島清隆.	膵がん細胞における恒常的高活性オートファジーによる CTL エピトープの産生	第 70 回日本癌学会学術総会(ポスター, #3204) 日本がん免疫学会総会プログラム・抄録集 pp499, 2011.	熊本	2011 年 10 月 5 日
赤塚美樹、松原亜以子、南谷泰仁、森島泰雄、高橋利忠、葛島清隆、小川誠司、恵美宣彦.	マイナー組織適合抗原をコードする一塩基多型のオンライン検索ツール	第 70 回日本癌学会総会 (ポスター, P-1444) 日本癌学会総会プログラム・抄録集 pp204, 2011.	大阪	2011 年 10 月 3 日

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Yukiya Yamamoto, Sachiko Tsuzuki, Yasushi Akahori, Yoshinori Ukai, Mariko Sumitomo, Masutaka Tokuda, Tadaharu Kanie, Akihiro Abe, Yoshiki Akatsuka, Yoshikazu Kurosawa, Nobuhiko Emi.	Isolation of human monoclonal antibodies directly modulating FLT3 signaling.	第 73 回日本血液学会学術集会(ポスター #PS2-284) 臨床血液抄録集 pp584, 2011.	名古屋	2011 年 10 月 15 日
赤堀 泰, 赤塚美樹, 葛島清隆, 恵美宣彦	HLA-A*02:01 拘束性に提示されたマイナー抗原 HA-1H ペプチドを認識する抗体の単離	第 4 回造血器腫瘍免疫療法研究会 プログラム抄録集抄録集 pp64.	金沢	H24 年 8 月 18 日
赤堀 泰, 稲熊容子, 赤塚美樹, 山本幸也, 村山裕子, 伊庭佐知子, 遠藤明美, 平松可帆, 葛島清隆, 恵美宣彦.	HLA-A2 拘束性に提示されたマイナー抗原 HA-1H ペプチドを認識する抗体の単離とその臨床応用に向けての検討 (口演 11-3).	第 35 回日本造血細胞移植学会 日本造血細胞移植学会総会プログラム・抄録集 pp202.	金沢	2013 年3月8日
Yoshiki Akatsuka, Hirofumi Taji, Yasuo Morishima, Koichi Miyamura, Yoshihisa Kodera, Nobuhiko Emi, Toshitada Takahashi, Tomohiro Kinoshita, Kiyotaka Kuzushima.	Vaccination With Minor Histocompatibility Antigen-Derived Peptides In Post-Transplant Patients With Hematological Malignancies - Preliminary Results.	2nd International Workshop on the Biology, Prevention, and Treatment of Relapse After Hematopoietic Stem Cell Transplantation. Abstract P-11 (pp34).	NIH Bethesda, MD, USA.	2012 年 11 月 6 日
下嶋典子、吉岡 聡、菱澤方勝、大森勝之、Geraghty DE、二戸辰夫、石谷昭子.	成人 T 細胞白血病ウイルス感染細胞株における HLA-F の発現解析.	第 19 回日本組織適合性学会	東京	2010 年 9 月 17－19 日

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吉岡 聡、一戸辰夫、下嶋典子、菱澤方勝、大森勝之、Geraghty DE、石谷昭子、高折晃史.	成人T細胞白血病ウイルス感染者のT細胞表面におけるHLA-Fの発現についての検討	第20回日本組織適合性学会	静岡	2011年8月28－30日
吉岡 聡、一戸辰夫、下嶋典子、菱澤方勝、大森勝之、Geraghty DE、石谷昭子、高折晃史.	成人T細胞白血病ウイルス感染者の末梢血T細胞におけるHLA-Fの表面発現分画についての検討	第21回日本組織適合性学会	東京	2012年9月15－17日
Ichinohe T.	Microchimerism-associated toler- ance to noninherited maternal antigens (NIMAs) reduces severity of GVHD after MHC-mismatched hematopoietic cell trans- plantation by a CD4+CD25+ T-cell-dependent mechanism.	The 16th Annual Summer Meeting of the Korean Society of Blood and Marrow Transplantation.	Busan, Korea	August 19, 2011.
Ichinohe T	Emerging roles of non-inherited maternal alloantigens (NIMAs) and inherited paternal alloantigens (IPAs) in HLA- mismatched hematopoietic cell transplantation.	The Joint Meeting of the 17th International Symposium on Gnotobiology and the 34th Congress of the Society for Microbial Ecology and Disease.	Yokohama, Japan	November 21, 2011.
Iida M, Kanda Y, Toubai T, Nakase K, Mitamura M, Kanda J, Fukuda T, Miyamura K, Kanamori H, Mori T, Iida H, Atsuta Y, Morishima Y, Sakamaki H, Ichinohe T	on behalf of the Hematopoietic Stem Cell Transplantation from Foreign Donors Working Group of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Unrelated Hematopoietic Stem Cell Transplantation from Foreign Donors: Current Status in Japan.	16th Congress of Asia Pacific Blood and Marrow Transplantation	Sydney, Australia.	October 30-31, 2011
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Ishii S, Ikegame K, Kaida K, Yoshihara S, Okada M, Kato R, Inoue T, Tamaki H, Fujimori Y, Soma T, Ogawa H.	A novel regimen of unmanipulated HLA-haploidentical transplantation using a small dose of anti-T-lymphocyte globulin for patients in high tumor burden.	The 54th annual meeting of the American Society of Hematology	Atlanta, USA.	2012, 12.8-11
Ikegame K, Taniguchi Y, Yoshihara S, Kaida K, Taniguchi K, Ishii S, Inoue T, Kato R, Okada M, Tamaki H, Fujioka T, Soma T, Ogawa H.	From murine model to clinical trial of graft-versus-GVHD, a second transplantation from another donor for the rescue from refractory acute GVHD.	2013 Tandem BMT Meetings	Salt Lake City, USA.	2013, 2.13-17
Shinichi Ishii, Kazuhiro Ikegame, Katsuji Kaida, Ruri Kato, Takayuki Inoue, Satoshi Yoshihara, Masaya Okada, Toshihiro Soma, Hiroyasu Ogawa	HLA-haploidentical myeloablative stem cell transplantation using anti-T-lymphocyte glibulin	第 74 回日本血液学会学術集会	京都	2012 年 10 月 20 日

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池亀和博, 海田勝仁, 石井慎一, 吉原哲, 谷口享子, 加藤るり, 井上貴之, 岡田昌也, 相馬俊裕, 小川啓恭.	血縁 HLA 半合致ミニ移植(haplo-mini)の他施設前向き臨床試験(第 / 相試験)	第 35 回日本造血細胞移植学会総会	金沢	2013.3
Ogawa H	Haplo-identical HCT from family members. Haplo-identical HCT from family members.	The 1st international scientific symposium on hematopoietic stem cell transplantation in emerging countries.	Hanoi, Bietnam.	2011, 11.10-12
Ogawa H	(JSA-EHA Joint Symposium – Stem Cell Source) Unmanipulated HLA-haploidentical stem cell transplantation	第 73 回日本血液学会学術集会	名古屋	2011.10.15
井上貴之、池亀和博、吉原 哲、海田勝仁、谷口享子、玉置広哉、藤岡龍哉、岡田昌也、加藤るり、山本庸子、相馬俊裕、小川啓恭	Host regulatory T cells contribute to the regulation of GVHD in murine MHC haploidentical BMT models	第 73 回日本血液学会学術集会	名古屋	2011.10.14
Kaida K, Ikegame K, Yoshihara S, Taniguchi K, Ishii S, Kato R, Inoue T, Okada M, Tamaki H, Fujioka T, Soma T, Ogawa H.	Unmanipulated HLA-haploidentical (2-3 antigen-mismatched) stem cell transplantation using myeliablative or reduced-intensity preconditioning regimen.	The 53th annual meeting of the American Society of Hematology	San Diego, USA.	2011, 12.10-13
	Presidential Symposium, Cord blood stem cell transplantation: from the bench to the bed, Intra-bone marrow transplantation of unwashed cord blood using reduced-intensity conditioning treatment.	第 34 回日本造血細胞移植学会	大阪	2012.2.24

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Inoue T, Ikegame K, Yoshihara S, Kaida K, Taniguchi K, Tamaki H, Fujioka T, Okada M, Kato R, Yamamoto Y, Soma T, Ogawa H.	Mechanism of marked reduction in the severity of graft-versus-host disease by reduced-intensity conditioning in murine MHC-haploidentical BMT model.	The 52th annual meeting of the American Society of Hematology	Orlando	2010
R. Kato, H. Tamaki, K. Ikegame, S. Yoshihara, K. Kaida, T. Inoue, J. Nakata, K. Taniguchi, T. Fujioka, M. Okada, H. Ogawa.	One-HLA-antigen-mismatched related hematopoietic stem- cell transplantation using graft-versus-host disease prophylaxis with methylpredonisolone.	The 15th Congress of Asia-Pacific Blood and Marrow Transplantation	Phuket Thailand.	2010, 10. 29-31
Yoshihara S, Maruya E, Kaida K, Taniguchi K, Kato R, Inoue T, Fujioka T, Ikegame K, Tamaki H, Okada M, Soma T, Kusunoki K, Hayashi Y, Saji H, Ogawa H	High risk of graft rejection in cases with HLA anitibodies undergoing haploidentical SCT without TCD	第 72 回日本血液学会学術集会	横浜	2010 年 9 月 25 日(plenary)
高橋義行	難治性ウイルス感染症に対するウイルス抗原特異的細胞障害性 T 細胞(CTL)の対外増幅法の開発と臨床第1相試験	第 52 回日本小児血液学会総会 血液シンポジウム、移植・細胞療法の進歩	大阪	2010 年 12 月 18 日
高橋義行	造血幹細胞移植後難治性ウイルス感染症に対するウイルス特異的抗原特異的細胞障害性 T 細胞(CTL)の対外増幅法の開発と臨床第一相試験	第 34 回日本造血細胞移植学会総会 日本造血細胞移植学会・日本再生医療学会合同シンポジウム	大阪	2012 年 2 月 24 日
Yoshiyuki Takahashi	Virus specific cytotoxic T-cell therapy for drug resistant virus infection after hematopoietic stem cell transplantation,	第 54 回日本小児血液・がん学会総会 細胞療法シンポジウム	横浜	2012 年 12 月 1 日

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発表者氏名	演題名	学会名	会場	日時
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西森久和、前田嘉信、杉山暖子、小林孝一郎、山筋好子、門久幸代、谷本光音、竹内賢吾	ドナーの Th17 細胞と Th1 細胞が慢性 GVHD 発症に関与する	第 33 回日本造血細胞移植学会総会		2011/3
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VI. 研究成果による特許権等の
知的財産権の出願・登録状況

研究成果による特許権等の知的財産権の出願・登録状況

種　類	受付（識別）番号	出願日
国内特許	発明の名称：骨髄ドリル 発明人：池原進、白藤泰司、中村修二、下田信夫、佐渡克行 国：日本 特許収得日：2010.09.03 特許番号：4576587	2007.01.23
国外特許	発明の名称：骨髄細胞の採取装置及び骨髄針「BONE MARROW HARVESTING SET AND BONE MARROW HARVESTING NEEDLE」 発明人：池原進、中村修二、青木正人、沼澤正明、足立正一 国：カナダ 特許収得日：2010.07.13 特許番号：2454600	2002.08.08
国外特許	発明の名称：骨髄細胞の採取装置及び骨髄針「BONE MARROW HARVESTING SET AND BONE MARROW HARVESTING NEEDLE」 発明人：池原進、中村修二、青木正人、沼澤正明、足立正一 国：メキシコ 特許収得日：2011.01.13 特許番号：282792	2004.02.06
国外特許	発明の名称：骨髄細胞の採取装置及び骨髄針「BONE MARROW HARVESTING SET AND BONE MARROW HARVESTING NEEDLE」 発明人：池原進、中村修二、青木正人、沼澤正明、足立正一 国：欧州（ベルギー、ドイツ、スペイン、フランス、イギリス、イタリア、スウェーデン） 特許収得日：2010.10.06 特許番号：1421907	2002.08.08
国外特許	発明の名称：骨髄細胞の採取装置及び骨髄針「BONE MARROW HARVESTING SET AND BONE MARROW HARVESTING NEEDLE」 発明人：池原進、中村修二、青木正人、沼澤正明、足立正一 国：インド 特許収得日：2010.11.23 特許番号：244204	2004.01.28

VII.　研究成果の刊行物・印刷

STEM CELLS AND DEVELOPMENT
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ORIGINAL RESEARCH REPORT

Combination of Intra-Bone Marrow–Bone Marrow Transplantation and Subcutaneous Donor Splenocyte Injection Diminishes Risk of Graft-Versus-Host Disease and Enhances Survival Rate

Ming Shi,[1,*] Yasushi Adachi,[2,3,*] Yunze Cui,[1,4] Ming Li,[1] Zhexiong Lian,[5] Yuming Zhang,[6] Seiji Yanai,[2] Chieko Shima,[2] Yuichiro Imai,[2] and Susumu Ikehara[1]

The combination of allogeneic bone marrow transplantation (allo-BMT) and donor lymphocyte infusion (DLI) is a useful method for establishing donor chimerism and preventing a relapse of leukemia/lymphoma. However, there is a risk of inducing uncontrollable fatal graft-versus-host disease (GVHD). In fact, allo-BMT plus intravenous (IV)-DLI using donor splenocytes induces fatal GVHD in recipient mice. In this study, we examined the effects of the combination of intra-bone marrow (IBM)-BMT and the subcutaneous injection of donor splenocytes (SC-DLI) on the allo-BMT system. Recipient BALB/c mice were conditioned by sublethal irradiation (5 Gy), followed by IBM-BMT plus IV-DLI or SC-DLI in C57BL/6 mice. The IV-DLI group showed better engraftment of donor hemopoietic cells than the control group (without DLI) but showed fatal GVHD. The SC-DLI group, however, showed good reconstitution and mild GVHD. These results suggest that the combination of SC-DLI and IBM-BMT promotes the reconstitution of hemopoiesis and helps reduce the risk of GVHD.

Introduction

BONE MARROW TRANSPLANTATION (BMT) was initially developed as a cure for certain diseases of the hematopoietic system such as aplastic anemia, leukemia, and immunodeficiencies [1–3]. Since then, BMT has been widely used for the treatment of autoimmune diseases, solid malignant tumors, multiple myelomas, myelodysplastic syndrome, and so on [4–9]. Allogenic-BMT (allo-BMT) is becoming more common owing to the discovery of more effective immunosuppressants, more powerful antibiotics, antithymocyte globulin, and fractionated irradiation [10–12]. Recently, we developed a new and powerful BMT method: intra-bone marrow (IBM)–BMT [13]. In this method, donor bone marrow cells (BMCs) are directly injected into the recipient's bone marrow (BM). A much greater number of donor hemopoietic stem cells and mesenchymal stem cells can therefore be inoculated into the recipient BM than by conventional intravenous BMT (IV-BMT). This results in rapid reconstitution of donor hemopoietic cells and permits a reduction in radiation doses as a pretreatment for BMT [14–16].

Donor lymphocyte infusion (DLI) is often used after allo-BMT to prevent disease relapse in the setting of T-cell–depleted BMT or nonmyeloablative conditioning regimens. It is also a combined method to convert mixed chimerism to full donor chimerism [17,18]. Donor T cells injected intravenously during DLI are activated in the host's lymphoid tissues, which then migrate to the target tissues of graft-versus-host disease (GVHD) and then mediate the GVHD. DLI, which is used as the combined conditioning therapy for BMT, helps to reduce relapse rates. However, DLI-induced GVHD is always associated with an increase in therapy-related morbidity because of its uncontrollable and fatal characteristics [19–26]. A key challenge for DLI is to balance the positive and negative effects of donor T cells in order to optimize the outcome.

In mice, allo-BMT plus IV-DLI using donor splenocytes can induce fatal GVHD due to the donor T-cell infiltration and proliferation in the GVHD target tissues such as the liver, spleen, intestine, and skin [27]. In this study, we examined the localizable effects of donor T cells (splenocytes) by subcutaneous injection (SC) of donor splenocytes in the

[1]Department of Stem Cell Disorders, [2]First Department of Pathology, and [3]Regeneration Research Center for Intractable Diseases, Kansai Medical University, Moriguchi City, Osaka, Japan.
[4]JIMRO Co., Ltd., Takasaki, Gunma, Japan.
[5]Institute of Immunology and School of Life Sciences, University of Science and Technology of China, Hefei, China.
[6]Department of Pediatrics, Nanfang Hospital, Guangzhou, China.
*These two authors contributed equally to this work.

allo-BMT system using a preconditioning regimen (sublethal irradiation). Compared with IV-DLI plus IBM-BMT, the SC-DLI plus IBM-BMT group showed good reconstitution and only mild GVHD. The survival rate in the SC-DLI group was much higher than in the IV-DLI group. These results suggest that the combination of SC-DLI and IBM-BMT promotes reconstitution of hemopoiesis and helps to reduce the risk of fatal GVHD.

Materials and Methods

Mice

C57BL/6 mice (B6) and BALB/c were purchased from Shimizu Laboratory Supplies (Shizuoka, Japan). All the mice were maintained in a pathogen-free room, and 8–10-week-old male mice were used in the present studies. The university's committee for animal research approved all experiments.

Reagents

The antibodies used in this study were as follows: fluorescein isothiocyanate (FITC)-labeled anti-mouse H-2^b Ab, phycoerythrin (PE)-labeled anti-mouse H-2^d Ab, peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-mouse CD45 Ab, and anti-mouse CD3 Ab (BD Pharmingen, San Diego, CA). Lysing buffer (BD Pharmingen) was used for the lysis of erythrocytes. Collagenase type IV, used for hepatocyte isolation, was purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

Whole-body irradiation of recipient mice

Gamma-irradiation was delivered by a Gammacell 40 Exactor (MDS Nordion, Kanata, ON, Canada) with two ^{137}Cs sources. Recipient mice were irradiated with 6, 5, or 4 Gy, the day before BMT.

IBM-BMT

BMCs were flushed from the medullary cavities of the femurs and tibias of donor mice with phosphate-buffered saline (PBS). After gentle dissociation, the BMC suspension was filtered through a 70-μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ). The BMC suspension was then centrifuged and the supernatant was aspirated. The BMCs were adjusted to 3×10^9 per mL. The thus-prepared BMCs (3×10^7) were injected directly into the tibial cavity of the recipient mice via the intra-bone marrow route (IBM-BMT) the day after irradiation, as previously described [13]. Briefly, the mice were anesthetized and the area from the inguinal region to the knee joint was shaved. The tibia was gently drilled with a 26-G needle through the patellar tendon into the BM cavity. The BMCs ($3\times10^7/10\,\mu L$) were then injected into the BM cavity using a microsyringe (50 μL; Ito, Fuji, Shizuoka, Japan).

Donor lymphocyte infusion

Spleens were removed from donor B6 mice and then minced with scissors. Single cells were prepared by milling in steel mesh, followed by filtering through a 70-μm nylon mesh in PBS containing 2% fetal calf serum. After centrifugation, the pellets were suspended in 1×lysis buffer and kept for 15 min at room temperature for the lysis of erythrocytes. The erythrocyte-depleted splenocytes were adjusted to $5\times10^7/0.2\,mL$ (2.5×10^8 per mL) in PBS and were then injected intravenously into the tail vein in the IV-DLI group or subcutaneously in the back in the SC-DLI group. Figure 1 shows the experiment protocol, including the days for treatment.

GVHD analysis and scoring

The recipients were monitored daily for survival, and every 5 days for body weight changes and clinical signs of GVHD after BMT. The clinical scoring was based on 6 parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea. A severity scale of 0 to 2 was used for each parameter, with a maximum score of 12 (Table 1). Clinical signs early after transplantation due to radiation toxicity were not considered as the appearance of GVHD [28,29].

The carcasses of the recipients that had died or had been sacrificed at 3 months after BMT were kept in 10% formalin. Tissues from GVHD target organs (liver, intestine, and skin) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were observed using an Olympus BX41 light microscope (Olympus, Tokyo, Japan) with a UPlanFL N 20×/0.50 objective. An Olympus DP-25 color camera using DP2-BSW software was used to acquire the images.

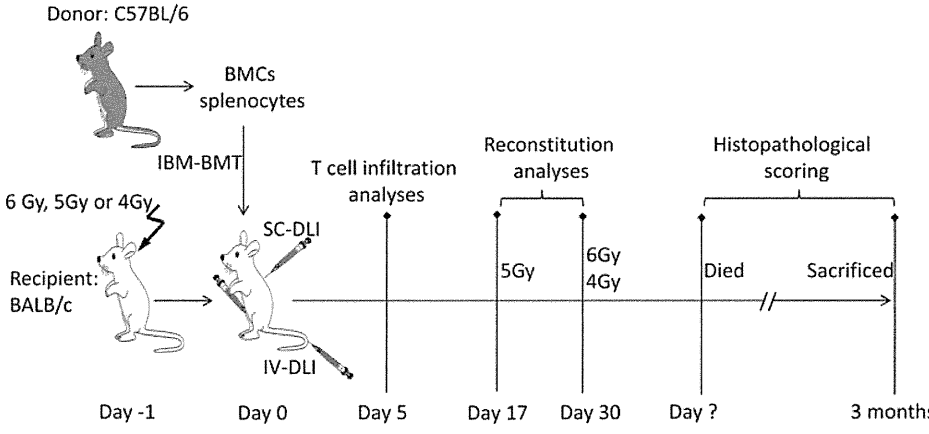

FIG. 1. Experiment protocol. The days of irradiation, BMT, and DLI are shown. The observed days of T-cell infiltration, reconstitution, and the analyses of histopathological scoring are also shown. BMT, bone marrow transplantation; DLI, donor lymphocyte infusion.

TABLE 1. CLINICAL SIGNS AND SCORING OF GVHD

Parameters	*Scale 0*	*Scale 1*	*Scale 2*
Weight loss	~10%	10%–25%	~25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mildly to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of non–hair-bearing skin	Obvious areas of denuded skin
Diarrhea	Normal	Bits of residual perianal fecal material	A mass of residual perianal fecal material

The clinical signs of GVHD (based on 6 parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea) were scored every 5 days after bone marrow transplantation. A severity scale of 0 to 2 was used for each parameter, with a maximum score of 12. GVHD, graft-versus-host disease.

Histopathological analysis was performed "single blind" by scoring the changes in the skin (dermal/epidermal lymphocyte infiltration, dyskeratotic epidermal keratinocytes, and epidermal thickening), the intestine (crypt regeneration, apoptosis in crypt epithelial cells, crypt loss, surface colonocyte attenuation, inflammatory cell infiltration in lamina propria, mucosal ulceration, and thickening of mucosa), and the liver (bile duct injury manifested by nuclear hyperchromasia, nuclear crowding, infiltrating lymphocytes, and cytoplasmic vacuolation and liver inflammation due to the infiltration of lymphocytes, neutrophils, and eosinophils). A severity scale from 0 to 4 was used, with a maximum score of 12 (Table 2) [29,30].

Analyses of donor cells in recipient peripheral blood, spleen, or liver by FACS

To detect the reconstitution of the recipients, the peripheral blood (PB) of the recipient mice was collected 17 days or 1 month after BMT. The PB was stained with FITC-labeled anti-H-2^b Ab, PE-labeled anti-mouse H-2^d Ab, and PerCP-Cy5.5-labeled anti-CD45 Ab. The erythrocytes were then lysed using lysing buffer. The stained cells were analyzed using FACScan (BD Biosciences, San Jose, CA). Leukocytes were first gated by $CD45^+$ cells, which were estimated as nuclear cells. The percentage of donor leukocytes was estimated as H-2^{b+}/$CD45^+$ cells.

To detect donor-derived T cells infiltrating the GVHD target tissues at 5 days after BMT, mononuclear cells (MNCs) from the recipient's spleen and liver were collected as follows: 0.5 mg/mL collagenase type IV solution was prepared by PBS dilution, and then after euthanasia, 2 mL collagenase solution was injected intraportally in the recipient mice. The spleen and liver were surgically excised and a single-cell suspension was prepared. The spleen and liver MNCs were then isolated by Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway). The MNCs were stained with FITC-labeled anti-H-2^b Ab, PE-labeled anti-mouse H-2^d Ab, and PerCP-Cy5.5-labeled anti-CD3 Ab. The percentage of donor T cells was analyzed by FACScan estimated as H-2^{b+}/$CD3^+$ cells.

Statistical analysis

Survival data were analyzed using the Kaplan–Meier method in the Stat Mate software. Other results are represented as means ± standard deviation (SD). The Student's *t*-test was used to determine any statistical significance. A P value of <0.05 was considered to be a significant difference.

Results

No GVHD occurs in the BMT-only group

Previous studies have shown that, in contrast to humans and other primates, no or only mild GVHD is observed in the

TABLE 2. HISTOPATHOLOGICAL GVHD SCORING

Organ	*Parameters*	*Scale 0*	*Scale 0.5*	*Scale 1*	*Scale 2*	*Scale 3*	*Scale 4*
Skin	Dermal/epidermal lymphocyte infiltration, dyskeratotic epidermal keratinocytes, and epidermal thickening	Normal	Focal and rare	Focal and mild	Diffuse and mild	Diffuse and moderate	Diffuse and severe
Intestine	Crypt regeneration, apoptosis in crypt epithelial cells, crypt loss, surface colonocyte attenuation, inflammatory cell infiltration in lamina propria, mucosal ulceration, and thickening of mucosa						
Liver	Bile duct injury, infiltrating lymphocytes, and cytoplasmic vacuolation and liver inflammation						

Histopathological GVHD scoring was performed based on the changes in 3 GVHD target organs: the skin, intestine, and liver. A severity scale of 0 to 4 was used for each organ, with a maximum score of 12.

case of allo-BMT without DLI in the murine setting [31–33]. To confirm this, BMCs from B6 mice were transplanted into sublethally irradiated (6, 5, or 4 Gy) BALB/c recipients by IBM-BMT (control group). All the control groups (6, 5, or 4 Gy) survived until 90 days after BMT (Fig. 2). No severe complications, such as fatal GVHD, occurred in the BMT-only control group according to clinical observations (Fig. 4) and histopathological evaluation (Fig. 5).

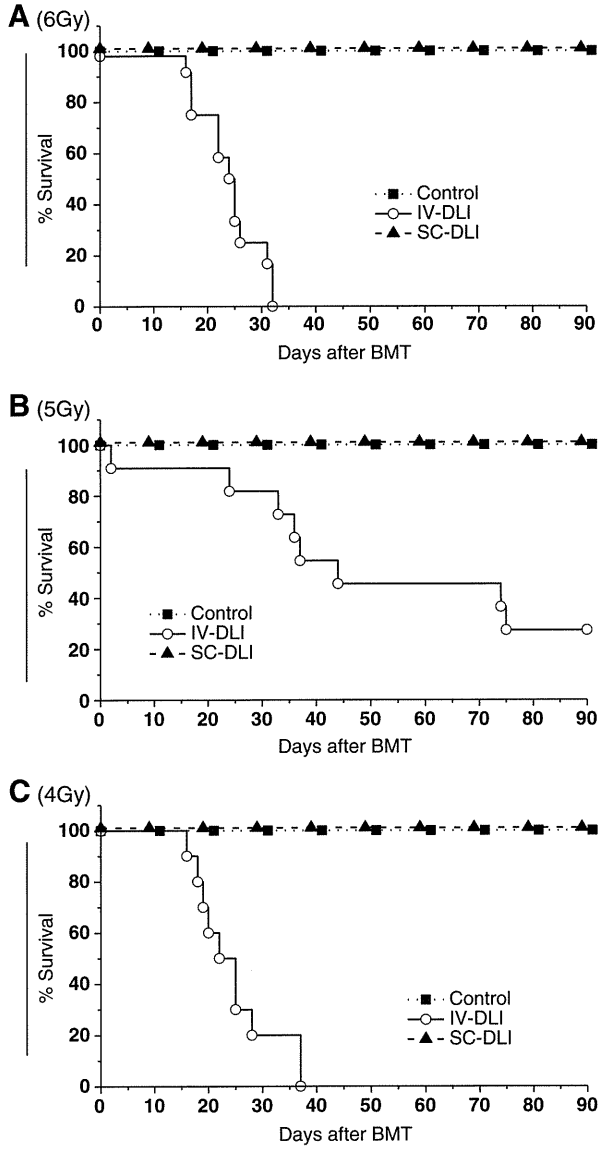

FIG. 2. IV-DLI shortens the survival but SC-DLI does not. Recipient (BALB/c) mice were irradiated at 6 Gy **(A)**, 5 Gy **(B)** or 4 Gy **(C)** on day −1. Bone marrow cells (3×10^7) from donors (B6) were injected by intra-bone marrow–BMT with or without 5×10^7 splenocytes DLI on day 0. The splenocytes were injected intravenously into the tail vein in the IV-DLI group or subcutaneously in the back side in the SC-DLI group. $n \geq 10$. IV, intravenous; SC, subcutaneous.

The SC-DLI group shows lower mortality than the IV-DLI group

The PB and spleen are commonly used as the source of lymphocytes for DLI. In the present study, we carried out DLI using the erythrocyte-depleted splenocytes (5×10^7 per mouse). After BMT, the recipients were monitored daily for survival. For the 6 Gy irradiated mice, all the mice in the IV-DLI group died within 32 days after BMT (Fig. 2), whereas all the mice in the SC-DLI group survived until 90 days after BMT. Similarly, high mortality rates were also observed in the IV-DLI group for 5 or 4 Gy irradiated recipients. To our surprise, the 5 Gy IV-DLI group showed significantly improved survival compared with the 4 Gy IV-DLI group. In contrast to the high mortality caused by IV-DLI, the SC-DLI groups (6, 5, or 4 Gy) showed a 100% survival rate, as seen in the control groups (Fig. 2).

Better reconstitution of donor hemopoietic cells is observed in both the IV-DLI and SC-DLI groups than the control group (without DLI)

Next, we examined the reconstitution degree of the recipients with donor-type cells in the PB of the recipient mice after BMT. In the experiment with preconditioning with 6 Gy irradiation, the PB was collected from the surviving recipient mice to analyze the chimerism at 1 month after BMT. All the recipients of IBM-BMT, either combined with DLI or not, showed nearly complete donor-type (H-2^d) hematopoietic cells (Fig. 3A). Moreover, there was no significant difference between the control group and the SC-DLI group: the mean and SDs of the percentages of donor hematopoietic cells were $92.4\% \pm 3.7\%$ and $95.3\% \pm 3.1\%$, respectively (Fig. 3D). We therefore reduced the radiation dose to 5 Gy. With 5 Gy, reconstitution was examined earlier (on day 17). Donor-type hematopoietic cells were detected in 4 of 10 control group recipients, 10 of 10 IV-DLI group recipients, and 9 of 11 SC-DLI group recipients (Fig. 3B). Statistically significantly higher percentages of reconstitution with donor cells were observed in the SC-DLI group than in the control group, and there was no significant difference between the IV-DLI and SC-DLI groups (Fig. 3E). Moreover, better reconstitution was confirmed by long-term (not transient) chimerism (data not shown). When the radiation dose was reduced to 4 Gy, neither the control group nor the SC-DLI group could reconstitute the recipients with donor BMCs. The two survivors in the IV-DLI group showed donor-type hematopoietic cells but these mice died soon (Fig. 3C, F).

Serious GVHD is observed in the IV-DLI group

After BMT, clinical signs of GVHD in the recipients (including weight loss, posture, activity, fur texture, skin integrity, and diarrhea) were assessed every 5 days and the score was calculated. In the 6 Gy IV-DLI group, all the recipient mice showed hunchback; several showed loss of weight, inaction, ruffled fur texture, and slight diarrhea on day 15. The signs of GVHD became progressively more serious: angular, severe hunching, stationary unless stimulated, severe ruffling, obvious areas of denuded skin, and severe diarrhea. The clinical score reached a peak on day 30: 7.33 ± 0.58. No obvious signs of GVHD appeared in the other

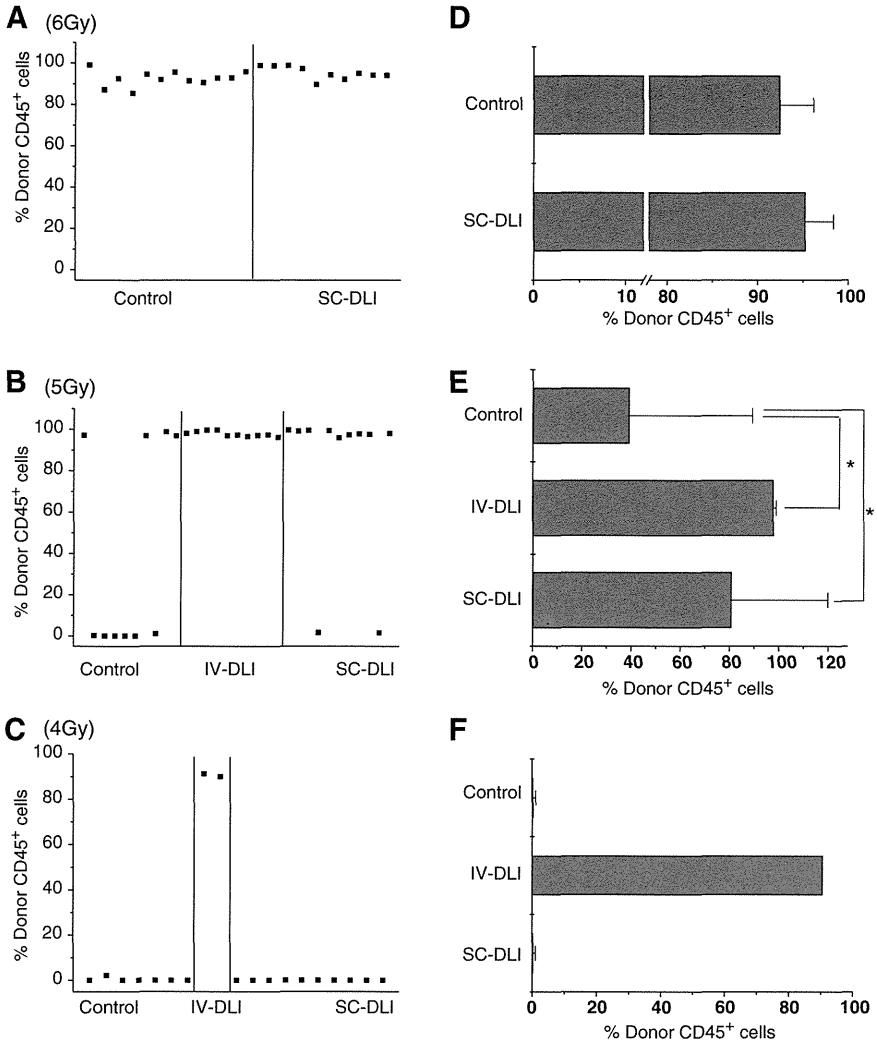

FIG. 3. Reconstitution in different irradiation doses. Recipient mice were irradiated with 4, 5, or 6 Gy on day −1. BMT and DLI were carried out on day 0. Peripheral blood of 6 Gy **(A** and **D)** and 4 Gy **(C** and **F)** irradiated mice was analyzed for reconstitution at 1 month after BMT. In the 5 Gy **(B** and **E)** experiment, peripheral blood was analyzed on day 17. Each small square (■) represents the percentage of reconstitution for each mouse in the left panels. The right panels show the percentage of donor $CD45^+$ by means ± standard deviation. $^*P <$ 0.05.

two groups: 0.25 ± 0.87 (control group) and 0.50 ± 0.71 (SC-DLI group) (Fig. 4A). In the 5 Gy experiment, the clinical scores in the control group, IV-DLI group, and SC-DLI group were 0.10 ± 0.31, 6.44 ± 2.60, and 0.81 ± 0.30, respectively, according to observations on day 30. The mice in the IV-DLI group retained the tendency to develop serious GVHD during the period from day 30 to day 70. The other two groups displayed mild GVHD (Fig. 4B).

In the 4 Gy experiment, the IV-DLI group displayed moderate but not severe GVHD, [although the recipient mice survived short-term (they died between days 16 and 37)]. Pathological diagnosis after autopsy indicated that infection due to graft failure (not GVHD) was the cause of death (data not shown).The clinical score was 4.00 (only two mice survived) on day 30 (Fig. 4C). It is not surprising that no GVHD was observed in either the control group or the SC-DLI group due to a failure of reconstitution.

GVHD is main cause of death in the IV-DLI group

We next examined the histopathological changes in the liver, skin, and intestine from randomly selected recipient mice that had died in the IV-DLI group or were sacrificed at 3 months after BMT in the control group and the SC-DLI group (four mice per group). The severity of histopathological GVHD nearly paralleled the clinical signs. In the IV-DLI group, either 6- or 5-Gy-irradiated recipients showed lymphocyte infiltration in the bile duct, epidermal thickening, and dermal lymphocyte infiltration, occasional crypt apoptosis, and mild inflammation in the intestine (Fig. 5A, C). The pathological scores in the IV-DLI group in the 6 and 5 Gy experiments were 5.75 ± 0.43 and 5.67 ± 0.47, respectively (Fig. 5B, D). In the control group and the SC-DLI group, no or only mild lesions were found in the GVHD target tissues, even in the skin at the site of the injection of splenocytes. The pathological scores in these two groups were also significantly lower than in the IV-DLI group in either radiation dose experiment. These data indicated that GVHD was the main cause of death in the IV-DLI group and that DLI via the SC route could diminish the risk of fatal GVHD.

SC-DLI reduces donor T-cell infiltration into GVHD target tissues

Previous studies reported that donor T cells reached a peak on day 5 after expansion and infiltration into target